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PREPROTEIN BINDING BY ATP-BINDING SITE MUTANTS OF THE *BACILLUS SUBTILIS* SecA

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The preprotein translocase of *Escherichia coli* (Wickner *et al.*, 1991) guards preproteins from the site of synthesis at the ribosome in the cytosol to the processed form to be released into the periplasm. SecA (Schmidt *et al.*, 1988) is the ATP-hydrolysing, peripheral subunit of the translocase (Brundage *et al.*, 1990; Wickner *et al.*, 1991), and plays an essential role in preprotein translocation (Lill *et al.*, 1989). The low endogenous ATPase activity of SecA is stimulated by interactions with acidic phospholipids, preproteins and the SecY/E protein (Lill *et al.*, 1989, 1990; Brundage *et al.*, 1990). This stimulated ATPase activity initiates translocation which is further driven by ATP hydrolysis and Δp (Schiebel *et al.*, 1991; Driessen, 1992). Biochemical studies suggest that SecA possess three ATP binding sites (Lill *et al.*, 1989; Oliver, 1993). Only one domain shows a significant level of sequence similarity to the Walker A- and B-motifs for a NTP-binding site (Walker *et al.*, 1982) (Fig. 1). Both regions are highly conserved among different bacterial and algal SecA homologues. To analyze the function of this putative ATP binding site, we started a site-directed mutagenesis approach and changed critical residues of the A-domain (Klose *et al.*, 1983; van der Wolk *et al.*, 1993) of the *Bacillus subtilis* SecA homolog (Overhoff *et al.*, 1991). Now we report on the localization of the B-domain and further characterized ATP- and preprotein-binding activities of the mutants.

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A-region B-region

Bacillus subtilis 96 NIAEMKTGEGKTLTSTLP 196 RPLHFAVIDEVDLSILIDEARTPLII
Escherichia coli 98 CIAEMRTGEGKTLTATLP 198 RKLHYALVDEVDLSILIDEARTPLII
Pavlova lutherii 94 KIAEMKTGEGKTLVAILP 194 NGFEFALIDEVDLSILIDEARTPLII
Anithamnion sp 90 KIAEMKTGEGKTLVAMLT 190 RDEFFALIDEVDLSILIDEARTPLII
 ****.*****.***
 GxTGxGKT RxxxxhhhhDEADxhh
 RxxxxhhhhDEADxhh

Fig 1. Sequence alignment of the putative ATP-binding domains of SecA homologs.

EXPERIMENTAL PROCEDURES:

Bacteria, plasmids and materials. Wild-type and mutant *B. subtilis* *secA* genes were cloned into the expression vector pTRC99A under regulatory control of the *trc* promoter/lac operator (Klose et al., 1993). *E. coli* strain JM109 (*recA1*, *endA1*, *hcr496*, *thi*, *hsdR17*, *relA1*, *supE44*, λ , $\Delta(lac-proAB)$, (*F'*, *traD36*, *proAB*, *lacIq* Δ MM15) harbouring the plasmids pMKL40, pMKL20, and pMKL21 (Table 1) was used for purification of SecA proteins as described (van der Wolk et al., 1993). pMKL18 contained the *E. coli* wild-type *secA* gene (Klose et al., 1983). SecA growth complementation experiments were performed with *E. coli* strains MM52 (MC4100, *sec451*^{ts}), BA13 (MC4100, *sec413*^{am}, *supF*^{ts}, *zch::Tn10*), MM54 (*geneX*^{ts}, *supF*^{ts}) and JM105.1 (*sec51*^{ts}, *leu::Tn10*, *thi*, *rpsL*, *endA*, *sbcB15*, *hspR4*, $\Delta(lac-proAB)$, [*F'*, *traD36*, *proAB*, *lacIq* Δ MM15]) (See Klose et al., 1993). ProOmpA and OmpA were isolated as described (See Driessen, 1992).

Proteolytic digestion. The conformation of SecA was probed by the sensitivity to Staphylococcal V8 protease (Shinkai et al., 1991). Reaction mixtures (50 μ l) contained: 60 μ g of SecA, 50 mM TrisCl (pH 8.0), 50 mM KCl, 5 mM MgCl₂ and 1 mM DTT. ATP (4 mM), (pro-)OmpA (640 μ g ml⁻¹), and/or *E. coli* phospholipid vesicles (800 μ g ml⁻¹) were added as indicated. Controls received 0.48 M urea. After 10 min preincubation at 37°C, 450 ng of V8 protease was added and aliquots (10 μ l) were withdrawn at different time intervals. Reactions were terminated with 5 μ l of blocking buffer (10 mM PMSF, 6 % SDS, 150 mM TrisCl [pH 6.8], 30% [v/v] glycerol, 15 mM DTT, and 0.03

% bromophenol blue) and heating for 5 min at 95°C. Samples were analyzed by SDS-PAGE.

Table 1. Plasmids

Plasmid	Amino acid substitution	<i>B. subtilis</i> <i>secA</i> allele	Reference
pMKL04	none	Wild-type	Klose et al., 1993
pMKL20/pMKL200	Lys ¹⁰¹ →Asn	K101N	Klose et al., 1993
pMKL21/pMKL210	Lys ¹⁰⁶ →Asn	K106N	Klose et al., 1993
pMKL4041	Asp ²⁰⁷ →Asn	D207N	M.Klose, unpublished
pMKL4040	Asp ²¹⁵ →Asn	D215N	M.Klose, unpublished

ADP binding. Binding of ADP to SecA was assayed using the fluorescent analog 1,N⁶-ethenoadenosine-5'-diphosphate (ϵ -ADP) (Molecular Probes, Eugene, OR). Solutions contained: 200 μ g ml⁻¹ of SecA, 50 mM TrisCl (pH 8.0), 50 mM KCl, 5 mM MgCl₂, 1 mM DTT and 10 μ M ϵ -ADP. Reactions received 5 mM of indicated nucleotides, 400 μ g ml⁻¹ proOmpA and/or 0.3 M urea. Fluorescence polarization measurements at 20°C were performed with an SLM 4800C (Aminco, Urbana, IL) fluorimeter equipped with Glan-Thompson polarizers. Excitation and emission was at 300 and 410 nm, respectively. The polarization (*P*) of ϵ -ADP fluorescence was calculated according to: $P = [(R_{\text{vert}}/R_{\text{horz}})-1] / [(R_{\text{vert}}/R_{\text{horz}})+1]$ where R_{vert} and R_{horz} are the ratios of parallel and perpendicular signals with the excitation polarizer placed in the vertical and horizontal orientation, respectively.

RESULTS:

Expression of B. subtilis secA A- and B-domain mutant genes in E. coli. Site-directed mutations were introduced in critical residues of the A- and B-domain of the putative catalytic ATP-binding site of the *B. subtilis* SecA (See table 1). Mutant proteins were expressed in various *E. coli* strains to test their ability to complement the growth-defects of conditional lethal *secA* mutations. After a shift to the nonpermissive temperature (42°C), full length, but non-functional SecA protein is synthesized in MM52 (Schmidt et al., 1988), a truncated amino-terminal SecA fragment is synthesized in BA13 (Cabelli et al., 1988), while due to a strong polar effect in the stop codon of *geneX*, no

SecA is synthesized in MM66 (Schmidt *et al.*, 1988). *E. coli* JM105.1 contains a single copy of the *lacZ^M* gene on the F' episome (Klose *et al.*, 1993). When the temperature sensitive strains MM52, BA13 or MM66 were transformed with plasmids pMKL40, wild-type *B. subtilis* SecA protein was found to complement the growth defects of these strains at the non-permissive temperature, provided that the protein is not brought to high expression level (Table 2) (Klose *et al.*, 1993). With pMKL210 and pMKL440 only poor growth was observed, even at the permissive growth temperature (Table 2). Lys¹⁰⁶ and Asp²¹⁵ therefore seem to be crucial for complementation of the *E. coli* secA mutant strains by the *B. subtilis* (this paper) and *E. coli* (M. Klose, unpublished) SecA.

Table 2. Growth complementation of *E. coli* secA mutants

secA Allele	Plasmid	<i>E. coli</i> strain									
		MM52		JM105.2		BA13		MM66			
		30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
pTRC99A		+	+	+	+	+	+	+	+	+	+
<i>B. subtilis</i>											
Wild type	pMKL40	+	+	+	+	+	+	+	+	+	+
K101N	pMKL200	+	+	+	+	+	+	+	+	+	+
K106N	pMKL210	(+)	-	(+)	-	(+)	-	(+)	-	(+)	-
D207N	pMKL441	+	+	+	+	+	+	+	+	+	+
D215N	pMKL440	(+)	-	(+)	-	(+)	-	(+)	-	(+)	-
<i>E. coli</i>											
Wild type	pMKL18	+	+	+	+	+	+	+	+	+	+
<i>E. coli</i> strains were grown on LB plates supplemented with 0.5 % glucose (G) or 1 mM IPTG (I). Growth temperatures were 30 or 42°C; growth; (+), poor growth; -, no growth.											

ATP enhances the V8 protease resistance of wild-type SecA, but not that of K106N SecA. Soluble *E. coli* SecA is sensitive to digestion by protease V8. ATP and the non-hydrolyzable analog AMP-PNP enhance the resistance of SecA against V8 digestion (Shinkai *et al.*, 1991), suggesting a conformational change upon binding of ATP. *B. subtilis* SecA proteins were purified and incubated with V8 protease. Wild-type SecA was rapidly digested by V8 protease, and was more resistant in the presence of ATP (Fig. 2A). K106N SecA was not protected against V8 protease digestion in the presence of ATP (Fig. 2B). These data suggest that Lys¹⁰⁶ is involved in an ATP-dependent conformational change of SecA.

Effect of proOmpA on V8 protease sensitivity of wild-type and K106N SecA. *B. subtilis* SecA ATPase activity is stimulated by proOmpA and the protein binds to translocation intermediates of proOmpA (van der Wolk *et al.*, 1993). On the other hand, K106N SecA blocks the Δp -dependent chase of such intermediates possibly through tight binding. Addition of proOmpA to the *B. subtilis* SecA, preincubated with ATP, restored the sensitivity to V8 digestion to the level when no ATP was present (Fig. 2A). A strong effect was observed with the K106N SecA, suggesting that this mutant binds proOmpA with greater affinity than the wild-type. OmpA had only little effect on the V8 protease sensitivity (not shown). These results demonstrate that K106N SecA interacts with preproteins.



Fig. 2. V8 protease sensitivity of wild-type (A) and K106N (B) SecA.

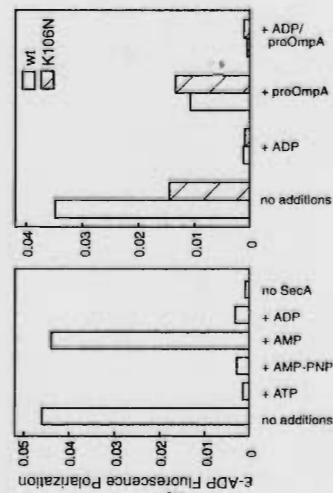


Fig. 3. Binding of ϵ -ADP to wild-type and K106N SecA.

Binding of ϵ -ADP to wild-type and K106N SecA. ϵ -ADP was used to assay ADP binding to SecA through fluorescence polarization measurements (Fig. 3A). Bound ϵ -ADP was chased by an excess ATP, ADP or AMP-PNP, while AMP was ineffective. K106N SecA still binds ADP (Fig. 3B, hatched bars), although with lower specificity than the wild-type (open bars). ProOmpA elicits release of bound ϵ -ADP from the wild-type to the level found for the K106N SecA. ProOmpA had no effect on the binding of ϵ -ADP to K106N SecA, while further release required an excess ADP. These results suggest that binding of proOmpA to SecA lowers its affinity for ADP, while K106N SecA appears to be frozen in this low-affinity ADP-binding state.

DISCUSSION:

In a previous communication (van der Wolk *et al.*, 1983), we have shown that Lys¹⁰⁶ is an essential residue of the A-domain of the catalytic ATP-binding site. The *in vivo* complementation assays now identify Asp²¹⁵ as part of the B-domain. This site differs from the one suggested by Koonin and Gorbelenya (1992). Further work will involve the purification of this mutant in order to analyze its activities *in vitro*.

A conceptual model for the active site of SecA based on the structure of RecA which has been solved at 2.7 Å with bound ADP (Story and Steitz, 1992) is presented in Fig. 4. In RecA, the enzymatic mechanism of ATP hydrolysis involves activation of a water molecule by a glutamate residue for an in-line attack of the γ -phosphate-presented by the invariable lysine (Lys¹⁰⁶ in SecA) of the A-domain. The position of the glutamate residue in SecA is unknown. The γ -phosphate is positioned such that Mg²⁺, stabilized by the invariable aspartate (Asp²¹⁵) of the B-domain, bridges the oxygen atoms of the β - and γ -phosphates such that the phosphate oxygens, the Mg²⁺ ion and the O γ of threonine of the A-domain (Thr¹⁰⁷) all lie in the same plane. In addition, the γ -phosphate is in contact with the so-called C-domain, not yet identified in SecA. The site for preprotein binding may be formed upon interaction between the β - and γ -phosphates and residues of the C-region that lie adjacent to the phosphate-binding loop. In analogy with the RecA model, ATP hydrolysis is expected to destroy interactions between the nucleotide and residues of the C-region, resulting in a change in conformation of the

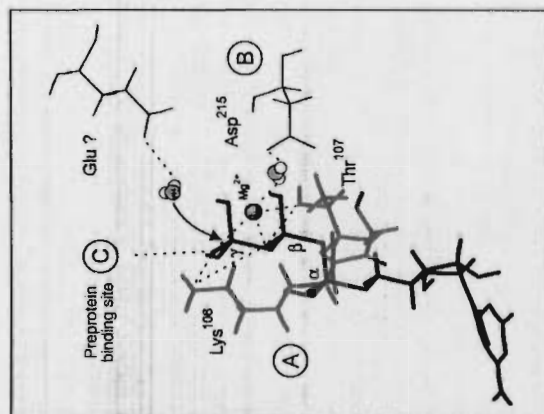


Fig. 4. Model for the SecA ATP-binding site.

translocation ATPase, while ATP antagonizes this inhibition (Cunningham and Wickner, 1989). Binding of the preprotein to SecA discharges the bound nucleotide possibly through lowering of the binding affinity (Shinkai *et al.*, 1991; this paper). This phenomenon is lost with the K106N mutant which binds ATP only with low affinity, maybe at an alternative low affinity binding site. We propose that the K106N SecA is prone to high-affinity preprotein binding. The inability to hydrolyse ATP (van der Wolk *et al.*, 1993) prevents the release of bound preproteins, thereby blocking preprotein translocation at the membrane translocation sites.

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Internal Disulfides in the Diphtheria Toxin A-Fragment Block Its Translocation to the Cytosol

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INTRODUCTION

A number of protein toxins from plants and bacteria efficiently kill eukaryotic cells by inhibition of protein synthesis. The toxin binds to receptors on the plasma membrane and is endocytosed, before the enzymatically active part of the toxin is translocated to the cytosol. Here it exerts its action by inactivating a crucial component of the cellular protein-synthesizing machinery.

The molecular mechanisms involved in the translocation process have been studied in most detail for diphtheria toxin, the main pathogenicity factor in clinical diphtheria (For reviews, see London, 1992, Sandvig and Olsnes, 1991). Diphtheria toxin is secreted as a polypeptide chain of 58 kD from *Corynebacterium diphtheriae*, and can easily be cleaved by trypsin into two fragments A (21 kD) and B (37 kD), joined by a disulfide bond. Diphtheria toxin entry into cells is initiated by binding of its B-fragment to specific surface receptors. Subsequently, the exposure to low pH in endosomes induces the translocation of the A-fragment to the cytosol. Translocation can also be induced at the level of the plasma membrane by exposing cells with surface-bound toxin to low pH. Low pH has been shown to induce unfolding and the exposure of hydrophobic regions in the toxin molecule, and this has led to the speculation that unfolding of the toxin may be necessary for the translocation to occur.

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